

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 December 2000 (07.12.2000)

PCT

(10) International Publication Number
WO 00/73347 A1

- (51) International Patent Classification⁷: C07K 16/12, G01N 33/569, 33/577
- (21) International Application Number: PCT/US00/14555
- (22) International Filing Date: 26 May 2000 (26.05.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/136,755 28 May 1999 (28.05.1999) US
- (71) Applicant (for all designated States except US): US GOVERNMENT as represented by THE SECRETARY OF THE NAVY [US/US]; A. David Spevack, Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, MD 20910 (US).
- (71) Applicants and
(72) Inventors: BURANS, James [US/US]; Chatam, NJ (US). ALDRICH, Jennifer [US/US]; Silver Spring, MD (US).
- (74) Agent: SPEVACK, A., David; Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, MD 20910 (US).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— With international search report.
— Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DIAGNOSTIC MONOCLONAL ANTIBODY FOR *YERSINIA PESTIS*

(57) Abstract: A monoclonal antibody reactive to Fraction 1 of *Yersinia pestis* and its use in detection of *Y. pestis* by antibody mediated assays such as the enzyme-linked immunosorbent assay and immunochromatographic, hand-held assays.

WO 00/73347 A1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION

TO ALL WHOM IT MAY CONCERN:

BE IT KNOWN THAT

5 **James P. Burans and Jennifer Aldrich**

residents of Chatham, New Jersey, and Chevy Chase, Maryland, respectively, and both citizens of the United States of America, have invented certain new and useful improvements in

DIAGNOSTIC MONOCLONAL ANTIBODY FOR *YERSINIA PESTIS*

of which the following is a PATENT SPECIFICATION

10 SPECIFICATION*Field of the Invention*

This invention relates to a monoclonal antibody which reacts with at least one surface antigen of *Yersinia pestis* and a method for detecting microorganisms in clinical samples by the use of the monoclonal antibodies produced by hybridoma cell lines, and more specifically to

15 methods for detecting *Yersinia pestis*.

Background Of The Invention

Plague is consistently reported in many countries in Africa, Asia and South America and the U.S.A. Confirmation and diagnosis of plague is typically predicated on the isolation and culture of *Yersinia pestis* leading to a retrospective identification. *Yersinia pestis* produces a

20 number of virulence factors encoded either chromosomally or on one of three plasmids. Fraction 1 (F1) capsular protein, unique to *Yersinia pestis*, enables the organism evade phagocytosis and is produced at 37°C but not below 27°C. Therefore, in the flea, and when first inoculated into humans, these organisms express F1 antigen and are easily phagocytosed, but not killed, by neutrophils and monocytes.

25 Because of the importance of the F1 antigen in plague virulence, standard enzyme-linked immunosorbent assays (ELISA) to F1 have been developed. Harte *et al.*, in US Patent No. 5,017,475, describe an ELISA using an unidentified mouse monoclonal directed against the F-1

antigen of *Y. pestis*. Monoclonal antibody reagents for use in ELISAs, compared with serum based antibody preparations, afford a number of advantages. Anti-sera prepared in animals are heterogeneous, unpredictable, and limited in supply. The advent of a classical technique to produce monoclonal antibodies (6) has allowed for the generation of immunodiagnostic reagents that are highly specific, homogeneous, and capable of being produced in large quantities. A simplified technique based on the Kohler and Milstein methodology for creating hybridomas (6), that produce monoclonal antibodies has been described (3), but a dependable standard monoclonal is needed.

Summary of the Invention

It is an object of this invention to provide a monoclonal antibody reagent for use in antibody based assays for *Yersinia pestis*.

It is another object of this invention to provide an immunological assay for the *Yersinia pestis* using a monoclonal antibody.

The fusion of a mouse myeloma cell line with a mouse lymphocyte creates a hybridoma cell line that produces a uniquely active monoclonal antibody. This antibody is specific and uniquely reactive for a bacterial antigen associated virulent strains of *Yersinia pestis*.

The uniquely active monoclonal antibody of the present invention is included in diagnostic reagents and methods used to detect and identify the causative agent of plague, *Yersinia pestis*.

These and other objects, features and advantages of the present invention are described in or apparent from the following detailed description of preferred embodiments.

Detailed Description Of The Invention

Mouse myeloma cell line, P3X63Ag8.653 ATCC CRL 1580, fused with *Y. pestis* F1 antigen immunized BALB/c mouse lymphocytes created a hybridoma which produced a uniquely active monoclonal antibody. This hybridoma is deposited with the American Type Culture Collection (ATCC) as ATCC _____ under the terms and conditions of the Budapest treaty for a period of thirty (30) years. Under the terms of the deposit, access to the culture will be

available during pendency of the patent application to individuals determined by the US Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. 122. Restrictions on the availability to the public of the culture will be irrevocably removed upon grant of the Patent.

5 The hybridoma techniques used in this investigation were similar to those reported by Kohler and Milstein, 1980 (2) as modified by de St. Groth and Scheidegger, 1980 (3).

 The unique antibody produced by the hybridoma is directly or indirectly linked to a detectable labeling group. Indirect linkage to a detectable label is achieved using biotin streptavidin technology or an anti-immunoglobulin. Other detectable labels that are employed in
10 the present invention include enzymes, fluorescent labels, colored latex particles, colloidal gold and radionuclides. The preferred label is an enzyme; colored latex; or colloidal gold particle that binds to the antibody at a position which does not interfere with the binding of the antibody to the antigen. The enzyme used for labeling possesses reactive groups to which the antibody is coupled without destroying enzyme activity and the enzyme does not occur naturally to an appreciable
15 extent in the type of sample to be assayed for the biological substance. Furthermore, the enzyme has a relatively long shelf life, a high specific activity and also is capable of being easily assayed, for example, with a visible light spectrophotometer. Parameters affecting enzyme labels will not effect signals generated by a colored particle such as latex or colloidal gold. The preparation of the enzyme-labeled or particle labeled antibody for use in the present invention can take place via
20 conventional methods known in the art.

 Examples of the coupling of biological substances to enzymes were described in Steinberger, 1974 (4). Examples of the latex and colloidal gold-labeled antibodies were reviewed by Gribnau *et al.*, 1986 (5). Specifically reviewed was colloidal gold labeling by Leuvering *et al.*, 1980 (6). These descriptions are incorporate herein by reference.

25 Indirect linkage of antibodies to detectable labels is accomplished with avidin-biotin technology. The use of primary-secondary antibody techniques in tandem with avidin-biotin technology affords an assay with increased sensitivity.

Having described the invention, the following examples are given to illustrate specific applications of the invention including the best mode now known to perform the invention.

EXAMPLE 1

Production Of Monoclonal Antibody Specific for *Yersinia pestis*.

5 **a. Immunization**

Female BALB/C mice were inoculated subcutaneously, each with 100-200 :g *Yersinia pestis* F1 antigen in 0.15 ml PBS diluted with 0.15 ml of Complete Freud's Adjuvant. Three further immunizations, administered at 14 day intervals with subcutaneous injections of 50-100 :g of *Yersinia pestis* antigen in 0.15 ml PBS diluted with 0.15 ml of Incomplete Freud's Adjuvant.

10 Following the last immunization, sera titers in a direct enzyme-linked immunosorbent assay ("ELISA") had reached sufficient levels to the *Y. pestis* antigen to perform a fusion. A final intravenous immunization containing 2.5 :g of *Y. pestis* antigen in 0.1 ml of PBS was delivered 24 hours before fusion.

b. Hybridoma Fusion

15 The P3X63-Ag8U.1 (653) mouse myeloma cell line was used for hybridoma fusions. The myeloma cells were grown to log phase and fused with donor splenocytes using pre-evaluated (polyethylene glycol) PEG 4000 (50% in Dulbecco's phosphate buffered saline (DPBS)). A mixture of myeloma cells and splenocytes were treated for one minute with the PEG mixture diluted with 9 ml of RPMI medium 1640 containing 1% penicillin/ streptomycin (P/S). The cells
20 were then centrifuged at 1300 rpm for eight minutes. The cell pellet was resuspended in HAT medium (Iscove's IMDM culture medium with 18% FBS, 1% Sodium pyruvate, 1% P/S, 1% L-glutamine, 1X HAT supplement (hypoxanthine, aminopterin, and thymidine, Sigma), and 1x HT media supplement (hypoxanthine, and thymidine, Sigma). The re-suspended mixture was
25 dispensed in 100 -l ml aliquots to wells of 96-well plates. HAT medium in 100 -l aliquots was added to each well. Plates were grown at 37°C, with 6% CO₂. Culture fluid was removed from the 96 well plates on day 14 and the media replaced with HT Medium. The tissue culture fluids from cultures with growing hybridomas were collected and assayed by ELISA for antibody to *Y.*

pestis F1 antigen. Cells from ELISA positive wells were transferred to 24 well plates and cultured to confluency. The cells were then cloned by limiting dilution. Positive wells were recloned a second time by limiting dilution.

c. **ELISA for Determination of Positive Clones and Subsequent Cloning by Limiting Dilution**

Separate rows of Immulon 2, 96 well plates were coated with up to 10-100ng/well of *Y. pestis* F1 antigen, as positive antigen coating, and 1.25µg/ml Bovine serum albumin (BSA) as negative antigen diluted in Coating buffer (0.01 M phosphate buffer, 0.15M NaCl, pH 7.4). Plates were incubated overnight at 4° C. Plates were washed six times with ELISA Wash Buffer, (PBS, 0.1% tween 20, 0.001% thimerosal, PH 7.4). Supernatants from clones were collected and added to positive and negative antigen coated wells. Normal mouse sera collected prior to the first immunization of the mice used for fusion and sera from the same mice post-immunization was also serially diluted (1:1 to 1:1,600). Antibody control reagents were diluted in ELISA Dilution Buffer (5% skim milk, 0.1% tween 20, 0.001% thimerosal, PBS, PH 7.4). Plates were incubated for 60 minutes at 37° C, and washed six times with ELISA Wash Buffer. Goat anti-mouse IgG, IgM and IgA horseradish peroxidase (HRP) conjugate, or other labels such enzymes, or radionucleotides, diluted 1:2500 in ELISA Dilution Buffer, PH 7.4 was added to ELISA plates. Plates were incubated for 60 minutes at 37° C, and washed six times with ELISA wash buffer. ABTS substrate was then added to each well. Plates were incubated for 30 minutes at 37° C. The optical densities (OD's) were read at a wavelength of 410 nm. Clones with OD readings of 0.500 and higher were considered positive, and transferred to 24 well plates for cloning, in Iscove's IMDM Cloning Medium (10% Origen, Hybridoma cloning factor Igen, 1% P/S, 1% Sodium pyruvate, 1% L-glutamine, and 18% FBS (Cloning Media)). Once cultures were confluent, supernatant was collected and tested by direct ELISA. All positive antibody producing clones were limit dilution cloned to a cell density of 3 cells per well in a 96 well culture plate. Cells were cloned in Cloning Medium. Supernatants were again tested by ELISA for antibody production, and transferred to 24 wells culture plates using cloning media. Positive clones

verified by ELISA were limit dilution cloned to 1 cell per well using Cloning Medium. Positive clones were transferred to 24 well cultures, once confluent, and then transferred to culture flasks.

Origen was gradually removed from the cell culture medium. To demonstrate specific antibody production, the supernatant culture fluids of final clones were tested a final time by direct ELISA.

- 5 Approximately 8 vials of each cell line were frozen in liquid nitrogen at a cell density of 2×10^6 cells/ml. The antibody containing supernatant of hybridoma clone F1-04-A-G1 were also tested for their specificity in reactivity with *Y. pestis* F1 antigen by direct ELISA. The results of these direct ELISAs are shown.

Reactivity of hybridoma F1-04-A-G1	
Antigen	Optical Density (A 410nm)
Staphylococcal Enterotoxin B	0.063
Ricin	0.047
<i>Y. pestis</i> F1 antigen	3.128
<i>Bacillus anthracis</i> spore	0.053
<i>Francisella tularensis</i>	0.044
<i>Vibrio cholerae</i> (0139)	0.076
Botulinum toxin	0.085
<i>Erwinia herbicola</i>	0.046
Bovine serum albumin	0.086

EXAMPLE 2

Antigen Capture ELISA

ELISA reagents are as described for Example 1. Alternating rows of Immulon 96 well
5 plates are coated overnight with 100 μ l per well of rabbit anti-plague IgG (Squibb) as positive
capture wells or for negative controls with normal rabbit IgG, at a concentration of 2.5 μ g/ml in
phosphate buffered saline (PBS), pH 7.4 at 4°C. The plates were washed six times with PBS
wash buffer, as described in Example 1. The plates were blocked with 150 μ l of ELISA blocking
buffer and incubated for 1 hour at 37°C. The plates are then washed six times with PBS wash
10 buffer. *Y. pestis* F1 antigen is added to the plates and diluted serially from 1.0 μ g/ml to 0.98 ng/ml
in ELISA dilution buffer. Each well contains 100 μ l of each antigen dilution. ELISA dilution
buffer with no antigen is added to 3 pairs of wells as negative controls. Unknown samples in 100
 μ l volumes are then added to duplicate wells. Plates are then incubated for 1 hour at 37°C. After
incubation, the plates are washed six times with PBS wash buffer and 100 μ l of 10 μ g/ml of the
15 monoclonal antibody F1-04-A-G1, diluted in ELISA dilution buffer, is added to each well. The

plates are incubated for 1 hour at 37°C. after which the plates are washed six times with PBS wash buffer. After washing, 100 µl of goat anti-mouse IgG (gamma specific) horseradish peroxidase (HRP) conjugate, or other suitable indicator, is then added to each well. The plates are then washed six times with PBS wash buffer and 100 µl of substrate is added to each well.

- 5 The plates are then incubated for 30 minutes at 37°C and the optical density of the liquid read at 410 nm. An example assay is illustrated in figure 1.

EXAMPLE 3

Use of anti-*Y. pestis* in rapid, hand-held, immunochromatographic assays.

- The assay system, essentially as described by Burans, *et. al.* (7), utilizes polyclonal antibody reactive to *Yersinia pestis* as a capture antibody, which is air brushed along a line on a nitrocellulose membrane, approximately 3 to 7 mm wide and 2 to 3 inches long which is resting on top of a plastic mounting strip. The membrane is then air dried and blocked briefly with polyvinylalcohol. Membrane strips with the monoclonal F1-04-A-G1, labeled with colloidal gold, is then mounted to overlap the blocked nitrocellulose membrane. An alternative procedure is to utilize polyclonal antibody as the colloidal gold detector and airbrush the monoclonal antibody F1-04-A-G1 onto the nitrocellulose. A sample delivery pad composed of cotton lint paper is then applied to overlap the lyophilized colloidal gold membrane strip. Another cotton lint membrane pad is then applied to overlap with the top of the nitrocellulose membrane. The total length of the membrane/pad construction is approximately 2.5 to 3 inches, which is then mounted into a plastic holder. Serum, body fluid or other liquid samples are added directly to the sample delivery pad. The fluid then "wicks" through the sample delivery pad, the lyophilized colloidal gold pad, the nitrocellulose membrane and then into the absorbent pad on the top of the strip. The assay is incubated for 15 minutes at ambient temperature. The appearance of a red line indicates a positive reaction.

REFERENCES

1. Anderson, George, Patricia Worsham, Christopher Bolt, Gerard Andrews, Susan Welkos, Arthur Friedlander and James Burans (1997) Am. J. Trop. Med. hyg. 56(4): 471.

2. Kohler, G. and Milstein, C. (1975) *Nature* 256: 495.
3. Fazekas DE ST. Groth, S., Scheidegger, D., (1980) *Journal of Immunological Methods*, 35:1.
4. Steinberger, L.A. (1974) *Immunocytochemistry*. Prentise Hall, New Jersey.
5. Gribnau, T.C.J., Leuving, J. H. W., van Hall, H., (1986) *Journal of Chromatography*,
376:175.
6. Leuvring, J. H. W., Thal, P. J. H. M., and van der Waart, M., (1980) *Journal of Immunoassay*,
1(1):77.
7. Burans, J., A. Keleher, T.O'brien, J. Hager, A. Plummer and C. Morgan. (1996) *Salisbury
Medical Bulletin (Special Supplement) No. 87:36*

10 The foregoing descriptions of the preferred embodiments are intended to be illustrative
and not limiting. It will be appreciated that numerous modifications and variations can be made
without departing from the spirit or scope of the present invention.

WHAT IS CLAIMED IS:

1. A monoclonal antibody which reacts with the Fraction 1 antigen of *Yersinia pestis*.
2. A monoclonal antibody according to Claim 1 wherein the monoclonal antibody is produced by
5 a hybridoma cell line resulting from the fusion of a mouse myeloma cell line selected from the group consisting of P3X63Ag8.653 ATCC CRL 1580 and P3X63-Ag8U.1 (653) with a BALB/c mouse lymphocyte.
3. A monoclonal antibody of Claim 1 wherein the monoclonal antibody is an IgG.
- 10 4. A diagnostic reagent comprising the monoclonal antibody of Claim 1 linked directly or indirectly to a detectable label.
5. A diagnostic reagent according to Claim 4, wherein the detectable label is selected from the
15 group consisting of an enzyme, a fluorescent marker and a radionucleotide.
6. A diagnostic reagent according to Claim 4, wherein the monoclonal antibody is linked to a detectable label via a secondary antibody reactive to the said monoclonal antibody of Claim 1 and bound to a detectable label.
- 20 7. A diagnostic reagent according to Claim 6, wherein the secondary antibody is covalently linked to a hapten and the detectable label is an antihapten.
8. A diagnostic reagent according to Claim 7, wherein said hapten is biotin and said antihapten is
25 selected from the group consisting of avidin and streptavidin.
9. A method for detecting *Yersinia pestis* comprising the steps of:

- (a) adhering *Yersinia pestis* F1 antigen to solid matrix;
- (b) contacting a media suspected to contain *Yersinia pestis* with said monoclonal antibody of Claim 1 linked to a detectable label; and
- (c) detecting the label, whereby the presence of *Yersinia pestis* in the media is determined.

5

10. A method for detecting *Yersinia pestis* comprising the steps;

- (a) contacting the media suspected to contain *Yersinia pestis* with the monoclonal antibody of Claim 1;
- (b) contacting the monoclonal antibody of Claim 1 with an antibody reactive to said monoclonal antibody and linked to a detectable label;
- (c) detecting the label, whereby the presence of *Yersinia pestis* in the media is determined.

10

15 11. A method for detecting *Yersinia pestis* comprising the steps;

- (a) adhering an antibody specific for *Y. pestis* to a solid matrix;
- (b) contacting a media suspected to contain *Yersinia pestis* with solid matrix containing antibody bound *Yersinia pestis*;
- (c) contacting the monoclonal antibody of Claim 1 to the solid matrix containing antibody bound *Y. pestis*;
- (d) contacting an antibody reactive to said monoclonal antibody and linked to a detectable label with the media suspected to contain *Yersinia pestis*; and
- (e) detecting the label, whereby the presence of *Yersinia pestis* in the media is determined.

20

25

12. A method for detecting *Yersinia pestis* comprising the steps;

- (a) adding the monoclonal antibody of Claim 1 that has been labeled with colloidal gold to a membrane mounted to a nitrocellulose membrane of an immunochromatographic assay;
- (b) contacting media suspected to contain *Yersinia pestis* with the monoclonal antibody of Claim 1 that has been colloidal gold labeled.

5

13. A method for detecting *Yersinia pestis* as in Claim 12 wherein the method is a hand-held assay.

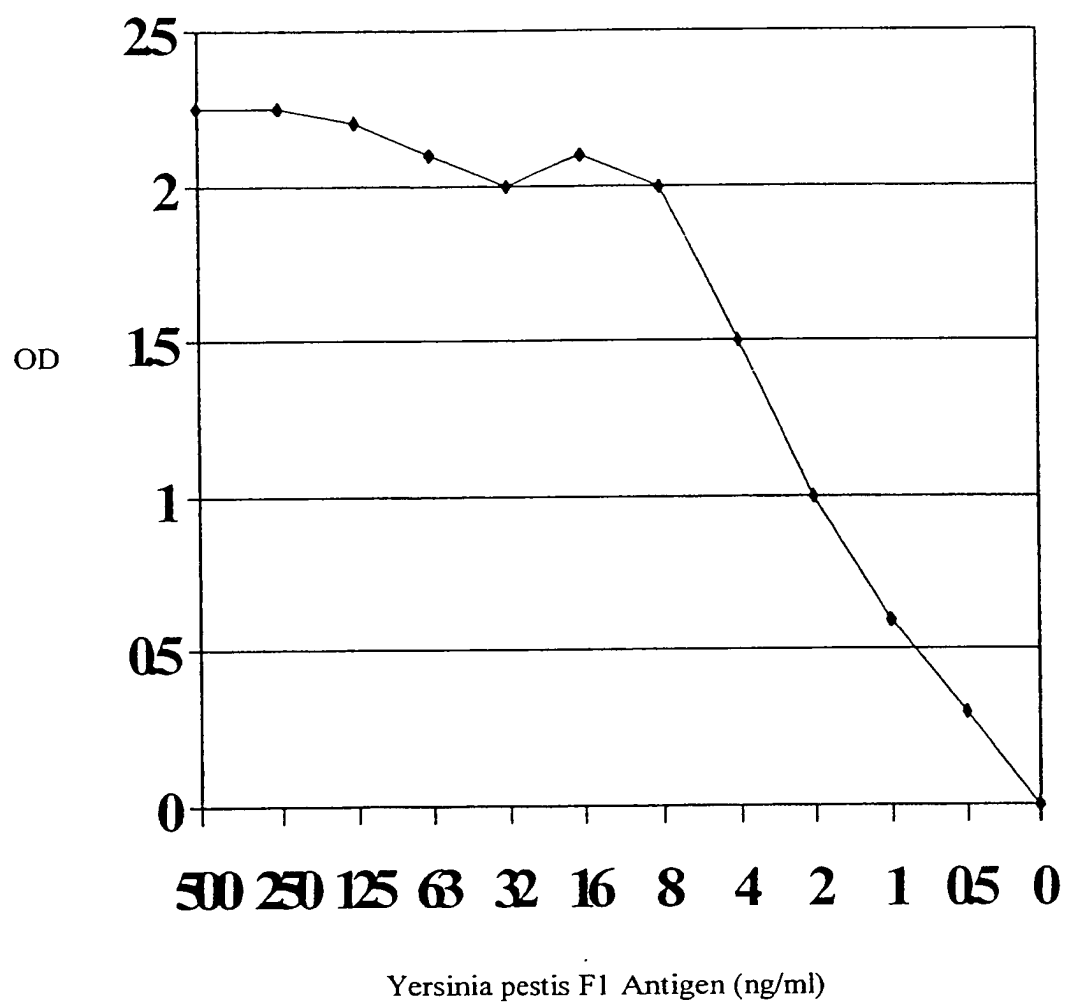


FIG.1/1

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/14555

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/12 G01N33/569 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WILLIAMS J E ET AL: "A MONOCLONAL ANTIBODY FOR THE SPECIFIC DIAGNOSIS OF PLAGUE." BULL W H O, (1988) 66 (1), 77-82. , XP000919061 abstract page 77, right-hand column, last paragraph -page 78, left-hand column, paragraph 2 ---	1-3
X	ANDERSON G P ET AL: "Assay development for a portable fiberoptic biosensor." ASAIO JOURNAL, (1996 NOV-DEC) 42 (6) 942-6. , XP000637786 page 944, right-hand column, last paragraph abstract --- -/--	1-13

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

13 September 2000

Date of mailing of the international search report

27/09/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

von Ballmoos, P

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/14555

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>ANDERSON G W JR ET AL: "Protection of mice from fatal bubonic and pneumonic plague by passive immunization with monoclonal antibodies against the F1 protein of Yersinia pestis."</p> <p>AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE, (1997 APR) 56 (4) 471-3. ,</p> <p>XP000918020</p> <p>page 471, left-hand column, last paragraph -right-hand column, paragraph 1</p> <p>---</p>	1,3
A	<p>LEUVERING J H ET AL: "Sol particle immunoassay (SPIA)."</p> <p>JOURNAL OF IMMUNOASSAY, (1980) 1 (1) 77-91. ,</p> <p>XP000918760</p> <p>cited in the application</p> <p>the whole document</p> <p>-----</p>	12